



**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:

Dario NERI et al.

Examiner: Alana M. Harris

Serial No.: 09/194,356

Group Art Unit: 1642

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Title: ANTIBODIES TO ED-B DOMAIN OF FIBRONECTIN, THEIR CONSTRUCTION  
AND USES

**DECLARATION UNDER 37 CFR §1.132**

I, Professor Kiyotoshi Sekiguchi declare as follows:

1. I am the same person who signed the "Sekiguchi" letters of record dated August 29, 2005 and December 22, 2005.

2. The following describes experiments which support the conclusions drawn in my prior letters. The following experiments were performed by me or under my supervision.

3. Standard microtiter well plates were coated with either the polypeptide fragment 7B89 (a human placental fibronectin fragment containing the fibronectin repeats 7, 8 and 9 and the ED-B domain) or with the entire ED-B domain containing placental fibronectin protein purified from human placenta ("whole fibronectin") (according Isemura et al., J.Biochem., 96: 163-169, 1984). Like 7B89, the latter, of course, also contains the mentioned repeats 7, 8, and 9, as well as the ED-B domain. For the purposes for which these two polypeptides are used in these experiments, they are fully equivalent to establish the scientific conclusions drawn herein.

4. Samples of the two antibodies deposited in conjunction with Japanese patent

applications JP(A) H2-76598 and JP(A) H4-169195 were obtained from storage facilities in my laboratory. OAL-CF525 was the antibody deposited in conjunction with the former application; and OAL-TFN-01 was the antibody deposited in conjunction with the latter application. (For convenience, these are referred to below simply as CF525 and TFN-01, respectively.) Also employed was the antibody L 19, provided by Dr. Luciano Zardi, one of the coinventors of the above-mentioned application. L 19 is well-established in the literature as binding directly to the ED-B domain of fibronectin. (Pini et al., J Biol Chem, 1998, 273, 21769-21776, Fattorusso et al., Structure, 1999, 7, 381-390, Santimaria et al. Clin Cancer Res, 2003, 9, 571-575).

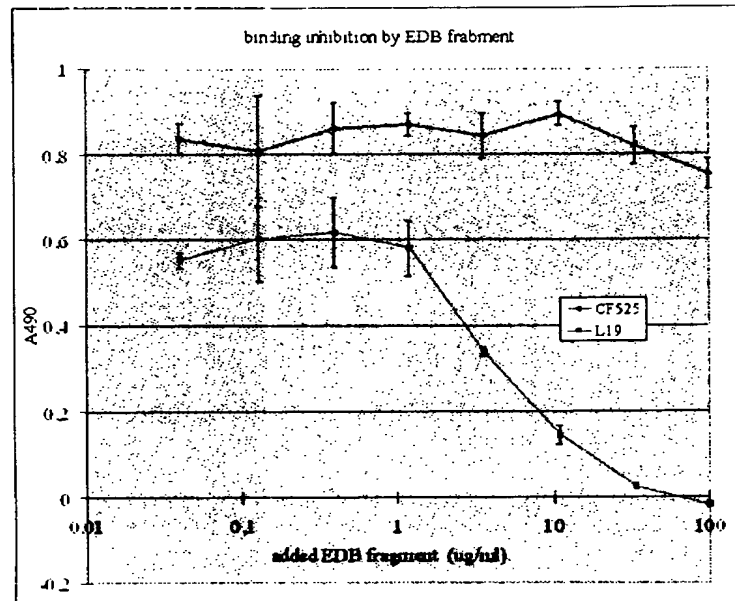
5 Standard ELISA assay procedures were used to conduct tests as follows. See the attached protocols also.

6. Experiment demonstrating lack of binding of CF525 to the ED-B domain of fibronectin.

The 7B89 fragment (a human placental fibronectin fragment containing the fibronectin repeats III 7, III 8 and III 9 and the ED-B domain between III 7 and III 8) was coated onto microtiter well plates using a solution of 1.5 µg/ml. Non-specific binding was blocked by treating the coated wells with 2% BSA/PBS (-) at room temperature for thirty minutes. Plates were then exposed to either (IgG) CF525 per se or (IgG) L19 per se at a concentration of 0.3 µg/ml. Plates were also exposed to the same two antibodies in the presence of various concentrations of recombinant ED-B fragment. In all cases, the wells were washed with 2% BSA/PBS (-), three times each. The wells were then exposed to a standard reporter antibody, peroxidase-conjugated goat anti-mouse IgG+IgM (H+L). This reporter antibody was designed to bind only to CF525 or L19 which has bound to the microtiter wells. Extent of binding was measured conventionally by detecting absorbance, A, at 490 nm.

The results are shown in the diagram below. As can be seen, the ED-B-specific antibody, L19 per se, binds to the fibronectin 7B89 fragment. However, pre-exposure of L19 to the ED-B fragment saturates the epitope of L19 by which it binds to the ED-B domain of the fibronectin fragment thus essentially eliminating its ability to bind to the fragment. This demonstrates the

known fact that L19 is specific for ED-B. In contrast, although CF525 also binds to the fibronectin 7B89 fragment when applied alone (because it binds to some epitope thereof), pre-treatment of CF525 with recombinant ED-B fragment has no significant effect on its binding to the 7B89 fragment of fibronectin. This demonstrates that CF525 is not specific for the ED-B domain of fibronectin.



### **Protocol for inhibition experiment of L19 and CF 525**

↓ Microtiter plates were coated with placental 7B89 (1.5 µg/ml) overnight

↓ Blocking by 2% BSA/PBS(-) at RT for 30min

↓ 1st Ab\* (either CF-525 or L19; 0.3 µg/ml) in the presence or absence of recombinant EDB fragment (0 µg/ml to 100 µg/ml) RT for 1 h

↓ Washed with 2% BSA/PBS(-), three times

↓ 2ndAb\*\* (0.1 µg/ml), RT for 1 h

↓ Detection

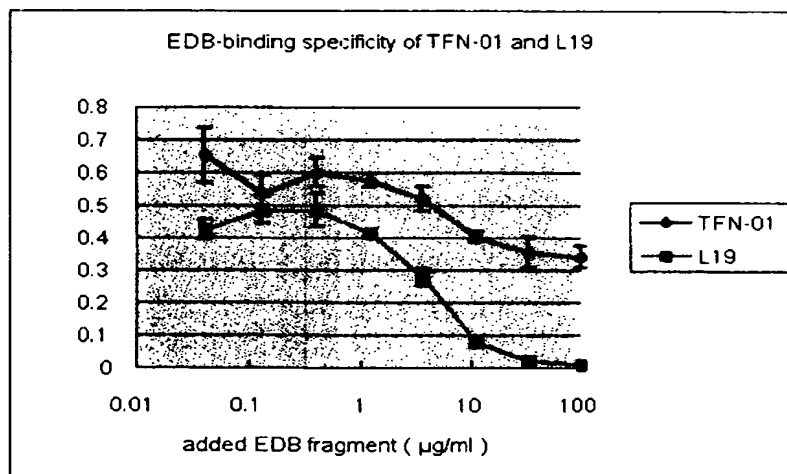
\*1st Ab used: CF-525 or L19

**\*\*2nd Ab used: Peroxidase-conjugated goat anti-mouse IgG+IgM(H+L)**

**Code Number:115-035-068(Jackson ImmunoResearch)**

7. Experiment demonstrating lack of binding of TFN-01 to the ED-B domain of fibronectin.

Whole fibronectin (defined above) was coated onto microtiter well plates using a solution of 5 µg/ml. Whole placental fibronectin was employed for TFN-01 ELISA's because it was originally screened for and defined against reactivity with placental fibronectin. Non-specific binding was blocked by treating the coated wells with 2% BSA/PBS (-) at room temperature for thirty minutes. Plates were then exposed to either (IgM) TFN-01 per se or (IgG) L19 per se at a concentration of 2 µg/ml. Plates were also exposed to the same two antibodies in the presence of various concentrations of recombinant ED-B fragment. In all cases, the wells were washed with 0.1% BSA/PBS (-), three times each. The wells were then exposed to a standard reporter antibody, peroxidase-conjugated goat anti-mouse IgG+IgM (H+L). This reporter antibody was designed to bind only to TFN-01 or L19 which has bound to the microtiter wells. Extent of binding was measured conventionally by detecting absorbance, A, at 490 nm. The results are shown in the diagram below.



### **Protocol for inhibition experiment of L19 and TFN-01**

↓Microtiter plates were coated with placental EDB-containing FN\*\*\* (5 µg/ml) overnight

↓Blocking by 2%BSA/PBS(-) at RT for 30min

↓1st Ab\* (either TFN01 or L19; 2 µg/ml) in the presence or absence of recombinant EDB fragment (0 µg/ml to 100 µg/ml) RT for 1 h

↓Washing with 0.1% BSA/PBS(-), three times

↓2ndAb\*\* (0.1 µg/ml), RT for 1 h

↓Detection

\*1st Ab used: TFN-01 or L19

\*\*2nd Ab used: Peroxidase-conjugated goat anti-mouse IgG+IgM(H+L)

Code Number:115-035-068(Jackson ImmunoResearch)

\*\*\*Placental FN was purified from human placenta according to Isemura et al. (J. Biochem., 96:163-169. 1984).

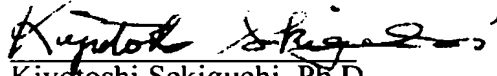
As can be seen, the ED-B-specific antibody, L19, binds to whole fibronectin. However, pre-exposure of L19 to the ED-B fragment saturates the epitope of L19 by which it binds to the ED-B domain of whole fibronectin, thus essentially eliminating its ability to bind to the fibronectin. This demonstrates the known fact that L19 is specific for ED-B. In contrast, although TFN-01 also binds to whole fibronectin when applied alone (because it binds to some epitope thereof), recombinant ED-B fragment has no significant effect on its binding to whole fibronectin. This demonstrates that TFN-01 is not specific for the ED-B domain of fibronectin.

For both CF-525 and TFN-01, the results obtained bear resemblance to the BC-1 antibody (Carnemolla et al., J Cell Biology. 1989, 108, 1139-48, Carnemolla et al. J Biol Chem, 1992, 267, 24689-92.) which was originally thought to bind directly to the ED-B and was then later unambiguously shown to recognize instead a cryptic (different) epitope in the oncofoetal fibronectin.

8. It will be noted that some of the conditions utilized in the foregoing experiments were different for the two tests. However, these differences simply reflect experimental design choices and have no influence on the validity or accuracy of the resultant data or the conclusions drawn herein.

9. Conclusion: The antibodies of my Japanese patent applications H2-76598 and H4-169195 do not seem to be specific for the ED-B domain of fibronectin.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

  
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